Co-administration of GP96 and Her2/*neu* DNA vaccine in a Her2 breast cancer model

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Abstract Heat-shock proteins have biochemical and immunological roles in chaperoning/signaling and activation of innate and adaptive immune responses, respectively. Their effect on the immune response is due to a phenomenon known as cross-priming of antigen, in which exogenous antigens are presented via MHC class I by antigen presenting cells. GP96 exerts adjuvant activity with some viral and bacterial antigens when applied in the form of a DNA vaccine. In this study, animals with Her2-expressing tumors were vaccinated by co-administration of GP96+ Her2/*neu* DNA vaccines. Analyses of the immune response, 2 weeks after the last immunization revealed decreased CD4⁺ CD25⁺ Foxp3⁺ naturally occurring regulatory T cells (Tregs) at the tumor site and increased IFN- γ /IL-4 level. Nevertheless, the graph of tumor size demonstrated a bi-phasic pattern in which partial control of tumor progression initially occurred, but finally its effectiveness was inversely affected by tumor size.

Keywords Her2 · GP96 · Regulatory T cells · Co-administration · DNA vaccine

Introduction

DNA vaccination is an illustration of vaccines that administer genes encoding the antigen, instead of the

antigen itself. Efforts to include cellular responses as well as antibodies expanded the idea of DNA vaccination. It is a means by which antigens are introduced into the MHC class I-processing pathway, a process known as cross-priming. This type of vaccination is promising for cancer immunotherapy. The genes coding for tumor associated antigens (TAA), such as carcinoembryonic antigen, Her2, and melanoma-associated antigens have been used in the form of DNA vaccines (Stan et al. 2006).

The Her2/ErbB2/*neu* proto-oncogene encodes 185 kDa (p185^{neu}) protein tyrosine kinase growth factor receptor and is a member of the epidermal growth factor receptor family. Over-expression along with cell surface localization and involvement in cancer progression/worsening prognosis has made this molecule as an ideal target of immunotherapy (Ishikawa et al. 1997; Pupa et al. 2005; Slamon et al. 1989). DNA encoding full-length or truncated is of protective and has therapeutic potential against Her2/*neu*-expressing mammary tumors (Rovero et al. 2000; Wei et al. 1999). However, the potency of Her2/*neu* DNA vaccine still needs to be improved. Some investigators co-administered constructs containing cytokine or co-stimulatory molecule genes and others utilized fusion technology to enhance the potency of Her2/*neu* DNA vaccines (Dela Cruz et al. 2005; Kim et al. 2005; Lin et al. 2004; Spadaro et al. 2005).

Heat shock proteins (HSPs) have so far been regarded as potent adjuvants in immunotherapy of cancers and infectious disease (Binder 2008). They are families of highly conserved and abundant intracellular proteins involved in chaperoning and many arms of the immune response (Srivastava 2002). GP96 (a member of HSP90 family), has been reported to also play an important role in innate and adaptive immune responses (Nicchitta 2003). Nicchitta et al. reported that GP96 could potentially stimulate maturation of antigen presenting cells and secretion of pro-

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inflammatory cytokines (Baker-LePain et al. 2004). Co-administration of the GP96 gene could produce a package which could simultaneously act as cytokine and co-stimulatory genes. GP96 effects on DNA vaccines containing some viral and bacterial antigens were previously studied (Bolhassani et al. 2008; Rapp and Kaufmann 2004). However, its role along with DNA vaccines encoding tumor antigens has not been fully elucidated. In this study, we co-administered the GP96 gene along with trans-membrane and extra-cellular domains (TM+ECD) of the rat Her2/*neu* and an immunological examination were performed to evaluate how GP96 affects tumor growth in this model.

Material and methods

Mice

Female BALB/c mice at the age of 8–10 weeks were purchased from Pasteur Institute, Tehran, Iran. Given free access to food and water, mice were housed for 1 week before experiments, and maintained in a good standard condition. All experiments were done according to Animal Care and Use Protocol of Tarbiat Modares University.

Cell line

TUBO is a cloned cell line derived from a lobular carcinoma that arose spontaneously from BALB/c mice transgenic for the rat HER2/*neu* (BALB-NeuT) oncogene. Professor Federica Cavallo (University of Turin, Turin, Italy) generously provided the TUBO cells. Rat Her2/*neu* is poorly immunogenic in normal BALB/c mice. As a xenogeneic protein, it does not induce anti-rat Her2/*neu* antibodies or any detectable cytotoxic T lymphocyte (CTL) response (Rovero et al. 2000). The cells were cultured in DMEM (CytoGen, Germany) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (BioSera Ltd, UK), and 20% heat-inactivated fetal bovine serum (Gibco BRL) and maintained at 37°C, 5% CO₂.

A suspension of 0.15 ml containing 3×10^5 of TUBO cells was injected subcutaneously. The cages were coded, and neoplastic masses were measured with calipers in the two perpendicular diameters. Vaccination was commenced 13 days after tumor implantation.

DNA expression vectors and vaccination

TM+ECD of Her2/*neu* (kindly provided by Professor Federica Cavallo; GenBank Accession No. NM_017003) was located between Hind III/Eco RI within pcDNA3* plasmid (pHer2). pcDNA3* was a derivation of the pcDNA3 plasmid (Invitrogen) by deleting the SV40

promoter, neomycin resistance gene, and SV40 poly(A) (Rovero et al. 2000). Human GP96 (kindly provided by Dr Brian Seed, Harvard Medical School, Boston, MA, USA; GenBank Accession No. NM_003299) was cloned into pcDNA3* between Xho I/Xba I sites (pGP96). Expression of each construct was evaluated by cloning of green fluorescent gene (GFP) at the end of gene (Chalfie et al. 1994; see below). All of the constructs were confirmed by sequencing.

Each construct was amplified using DH5α strain of *Escherichia coli* in Luria-Bertani medium. Large-scale preparation of the plasmid was performed according to standard polyethylene glycol precipitation method (Sambrook and Russell 2001). The animals were grouped into five cages and at day0 the tumor was implanted. Thirteen days later, the animals were intramuscularly vaccinated with 100 µg of DNA vaccine three times at weekly intervals with saline, empty plasmids, pHer2, or 50 µg pHer2+ 50 µg pGP96. Mice were sacrificed 2 weeks after the last vaccination. The mean diameter of tumor was recorded every other day from the beginning of vaccination until the animals were sacrificed.

In vivo gene expression

To confirm that in vivo expression of each construct occurred, gene expression was shown using murine muscle cells. The shaved point of injection was marked on the muscle and 25 µg of pHer2/GFP or pGP96/GFP plasmid or pcDNA3* in a volume of 60 µl was administered along the longitudinal axis of the mouse quadriceps muscle and parallel to the myofibers using a 28-gage needle and a 0.5 cc insulin syringe. Mice were sacrificed 11 days after plasmid DNA injection. The muscles were then removed, frozen in OCT tissue embedding medium, and sectioned (8 µm) with a cryostat. Sections were placed on slides and GFP expression was observed by direct fluorescence microscopy using an Axiphot Zeiss microscope and photographed with a digital camera.

Proliferation assay of spleen cells

An extract of TUBO cells was prepared by making a suspension of a tumor mass, passing through mesh, freeze/thawing, sonication, and finally filtering the extract. The concentration of the extract was then determined at 595 nm and stored frozen at −20°C until used.

Spleen cells from vaccinated animals in various groups were cultured for 24 h at 37°C in 5% CO₂ with 20 µg/ml the extract of TUBO cells (*T*, test) or PHA (*P*, positive control) and cells with no treatment (*N* negative control). The cells were then pulsed for 48 h with 5-bromo-2'-deoxyuridine (BrdU) labeling solution. Uptake of BrdU was detected using the cell proliferation enzyme-linked immunosorbent assay (ELISA) BrdU kit (Roche Diagnostic

GmbH, Mannheim, Germany) and expressed as stimulation index (SI):

$$SI = [(T - N)/(P - N)] \times 100$$

Interferon- γ and interleukin-4 production

Of the spleen cells, 1×10^6 were re-suspended in 1 ml of fresh RPMI-1640 containing 10% FBS and antibiotics and cultured at 37°C and 5% CO₂ for 90 h with 20 μ g/ml TUBO cell extract in 24-well plates. The plates were then centrifuged at 250 g for 10 min. Supernatants were then collected and frozen at -70°C, until the samples were analyzed to detect the presence of interferon- γ (IFN- γ) and interleukin-4 (IL-4) using a DuoSet ELISA system (R&D Systems) according to the manufacturer's instructions.

Flow cytometric analysis of the tumor CD4⁺/CD8⁺ and spleen/tumor CD4⁺CD25⁺Foxp3⁺ (Treg) populations

The solid tumors were cut into small pieces, minced with forceps and scalpel, rinsed twice with phosphate buffer saline (PBS), and passed through a 150- μ m stainless steel mesh. The mononuclear cells (MNCs) of the suspension were isolated by density centrifugation (700 $\times$ g, 15 min, and 20° C) using ficoll hypaque (Sigma, UK). Then the layer containing MNCs was removed, washed twice with PBS for 10 min in 360 g and 4° C, and the precipitated cells were re-suspended in PBS containing 2% fetal calf serum (Gibco, UK). The viability of the cells was determined using trypan blue (90%). Of the cells, 1×10^5 were labeled for CD4⁺ and CD8⁺ markers using FITC-conjugated anti-CD4 or anti-CD8 monoclonal antibodies or a fluorescein-conjugated anti-mouse IgG2a, as negative control (Becton Dickinson). Flow cytometric analysis was performed with a FACSCalibur (BD Bioscience).

Spleen and tumor MNCs were labeled for CD4⁺, CD25⁺, and Foxp3⁺ markers using a Mouse Regulatory T Cells kit, according to manufacturer's instructions (eBioscience, UK). The cells were fixed by 4% para-formaldehyde. Three-color flow cytometric analysis was employed with a FACS Calibur (BD Bioscience) and results were analyzed using WinMID 2.9 software (<http://scripps.edu/software.html>; Scripps Institute, La Jolla, CA).

Statistics

The significance of statistical comparisons was calculated using one way ANOVA and two-tailed Student's *t* test. Values of $P < 0.05$ were considered to represent statistically significant differences. Results are displayed as means $\pm$ standard deviations.

Results

In vivo gene expression

To investigate gene expression, we utilized GFP gene and clone GFP gene (Chalfie et al. 1994) before the stop codon located at the end of GP96 in pGP96 and Her2/*neu* in pHer2. Indeed, we attempted labeling of each gene so that each protein product of the construct was tagged with green fluorescent protein.

To test whether pHer2/GFP and pGP96/GFP constructs could be expressed in vivo properly, we used murine muscle cells as an in vivo environment. Constructs of pHer2/GFP and pGP96/GFP were transferred into murine muscle cells via direct injection. Eleven days later, muscle cells were removed to examine the expression of the gene products by direct fluorescence microscopy. Positive gene expression was observed in the cell sections (Fig. 1a and b). No fluorescent signal was observed from the control

Fig. 1 Photograph of in vivo gene expression, 11 days after injection of **a** pHer2/GFP or **b** pGP96/GFP observed by direct fluorescence microscopy

Fig. 2 Protocol of DNA vaccination. Thirteen days after tumor implantation, the animals were injected with DNA vaccine intramuscularly three times at weekly intervals. Two weeks after the last vaccination, immunological evaluation was performed

samples using injection of pcDNA3* (data not shown). Fluorescent dye due to Her2-GFP or GP96-GFP protein production in the transfected muscle cells was detected using fluorescence microscopy, confirming the successful construction and correct expression of the constructs in murine muscle cells. DNA sequence analysis also revealed that the Her2 or GP96 gene were located in frame with GFP gene.

Effect of DNA vaccines on established tumors

There is less than a 6% difference in the amino residues between rat Her2/*neu* and its surrogate in mice. It can be regarded as a xenogeneic resource of TAA (Nagata et al. 1997). TUBO cells grew progressively in BALB/c mice, as they are weakly immunogenic and the animal does not react against Her2/*neu* expressed by tumor cells. The tumor growth does not differ from those observed in BALB-neuT (Rovero et al. 2000). In this study, we assessed the effect of DNA vaccination pHer2, or pHer2+ pGP96 on the progression of established tumors in BALB/c mice induced

by TUBO cells. In addition, we investigated if the effect of Her2/*neu* DNA vaccine could be enhanced by co-administration of gp96 gene to the TM+ECD of rat Her2/*neu*. The protocol of the study from the beginning of subcutaneous tumor implantation and then to inoculation of DNA vaccine is shown in Fig. 2. The course of tumor growth is shown in Fig. 3. The pattern of tumor growth in animals vaccinated with pGP96+ pHer2 could be divided into two parts: part I, in which control of tumor growth in GP96+ Her2 group was partly better than the Her2 group. In part II, control of tumor growth in the Her2 group was partly better than GP96+ Her2 group.

Cellular reactivity

The enhancement of the cell-mediated mechanisms associated with the administration of pHer2, or pHer2+ pGP96 was evaluated using an in vitro proliferation test and measurement of IFN- γ /IL-4 levels.

Spleen cells were collected 40 days after tumor implantation and re-stimulated with the TUBO cell-raised tumor extract. The proliferative response of mice vaccinated with pHer2+ pGP96 was markedly enhanced in comparison to negative control ($P<0.001$) and pHer2-vaccinated animals ($P=0.007$). However, the index was also marginal increased in the pHer2-vaccinated animals, but it was not statistically significant (Fig. 4).

Spleen cells of the animals vaccinated with pHer2+ pGP96 plasmids induced a significant increase in IFN- γ responses when stimulated with TUBO cell-raised tumor extract, as compared to negative controls ($P=0.01$) and pHer2-vaccinated animals ($P=0.012$). However, vaccination with pHer2 did not induce a significantly different IFN- γ response in comparison to negative control. In contrast, animals vaccinated with pHer2 ($P=0.027$) or pHer2+ pGP96 ($P=0.003$) induced significantly higher IL-4-producing cellular immune responses (Fig. 5) in comparison to negative controls.

Fig. 3 Rate of tumor growth registered from the day before beginning of vaccination until 2 weeks after the last vaccination. Tumor masses were measured every other day with calipers in the two perpendicular diameters

Fig. 4 Proliferative response. Spleen cells were re-stimulated with the TUBO cell-raised tumor extract in the presence of BrdU. Results are shown as SI and represent the mean $\pm$ SD

Spleen and tumor CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells and tumor CD4 and CD8 T cell levels

No differences in the percentage of CD4⁺CD25^{high}Foxp3⁺ Tregs in the spleen of the various treatment groups were found. No change in Tregs percentage was found at the tumor site in mice vaccinated with pHer2, in comparison with the control mice. In contrast, a statistically significant decline in Tregs percentage at the tumor site was found in pHer2+ pGP96-vaccinated animals ($P=0.001$). The therapeutic effect appeared to be also Treg level-dependent, thereby demonstrating that Tregs percentage is decreased in the course of vaccination, consistent with better control of the progression of established tumors (Fig. 6a).

Determination of CD4⁺/CD8⁺ at the tumor site revealed a significant increase in CD4⁺ percentage in pHer2+ pGP96-vaccinated mice ($P=0.05$), in comparison with controls. CD4⁺ percentage in pHer2-vaccinated mice was only marginally increased ($P=0.1$). No significant change in CD8⁺ cells percentage was found at the tumor site in mice vaccinated with pHer2+ pGP96- or pHer2, as shown

Fig. 5 IFN- γ & IL-4 responses in DNA vaccinated mice. The level of responses was determined by a DuoSet R&D ELISA system after 2 weeks of the last immunization. Spleen cells were stimulated with TUBO cell-raised tumor extract in RPMI 1640 10% FBS for 90 h

in Fig. 6b. We found a reverse correlation between CD4⁺ and Tregs percentage (Fig. 6c).

Discussion

Many studies have attempted to use Her2 with an appropriate adjuvant to enhance Her2 vaccines. HSPs have

Fig. 6 Freshly isolated spleen cells and tumor MNCs were stained with anti-CD4 FITC, anti-CD25 PE, and anti-foxp3 PECy5. After gating on CD4⁺CD25⁺ cells, Tregs were defined as CD25^{high} and Foxp3⁺ (a). Tumor MNCs were labeled with FITC conjugated anti-CD4 or -CD8, freshly. CD4⁺ percentage was observed to significantly in pHer2+ pGP96- and marginal increase in the pHer2-vaccinated animals (b). We found a reverse correlation between Tregs and CD4⁺ percentage. As Tregs decrease, CD4⁺ cells increase at the tumor site (c)

been shown to be potent adjuvants in immunotherapy of tumors (reviewed by Binder 2008), since they function as “chaperokines”, i.e., chaperones+cytokines (Asea et al. 2000). The immunological importance of gp96 is its role in both the innate and adaptive immune systems. GP96 is a potent inducer of dendritic cells maturation. However, GP96 can stimulate secretion of pro-inflammatory cytokines by macrophages and elicit an NK response which is crucial against tumors (Srivastava 2002). Because DNA vaccination has also been described as a simple method which leads to protein expression and potent immune responses, we chose this approach. In this study, we constructed the eukaryotic expression vector containing TM+ECD of rat Her2/*neu* or GP96 gene (pHer2 and pGP96, respectively). The immune response triggered by co-administration of pHer2+pGP96 was evaluated and compared with pHer2, when administered intramuscularly to BALB/c mice with established tumor. Our results showed that administration of 100 µg pHer2 could partially control tumor progression, whereas co-administration of 50 µg pHer2+50 µg pGP96 was able to partly improve the pHer2 effect but its effect did not last to the end of study. The study indicates that co-administration of GP96 with Her2 can just partly control tumor progression and finally tumors progressed more than the group vaccinated with Her2 alone. Co-administration of 50 µgGP96+50 µgHer2 showed a bi-phasic pattern. In the first part, control of tumor growth in the GP96+Her2 group was better than in the Her2-vaccinated group, whereas in the second part control of tumor growth in Her2 group was better than the GP96+Her2-vaccinated group. It can be considered that in part I, with smaller tumor mass, GP96 could compensate the lower dose of Her2 or 50 µgGP96+50 µgHer2 could compete with 100 µgHer2. In the part two, with larger tumor mass, GP96 was not effective.

Administration of 50 µg pHer2+50 µg pGP96 led to significant proliferation of spleen cells. This is consistent with our previous results in which GP96 C- (Pakravan et al. 2010a) or N-terminal (manuscript under preparation) could cause spleen cells proliferation. However, it seems that co-administration of pGP96 (containing both C and N-terminal) with pHer2 had an additive effect on spleen cells proliferation.

In terms of IFN- γ /IL-4 level, there was an increase in IFN- γ level in the pHer2+ pGP96-vaccinated animals, but not in the pHer2-vaccinated group, in comparison with the control groups. The pattern of IL-4 level change was different. The animals vaccinated with pHer2+ pGP96 or pHer2 showed a significant rise at the IL-4 level, in comparison to the control groups. In addition, the groups vaccinated with pHer2+ pGP96 increased IL-4 level in comparison with pHer2-vaccinated mice. We previously (Pakravan et al. 2010a) demonstrated that fusion of GP96

C-terminal to Her2 could raise IL-4/IFN- γ level, which was concomitant with better control of tumor growth. Another study revealed that administration of HSP70 and Her2 (Pakravan et al. 2010b) led to tumor progression while IL-4/IFN- γ level was much more than that of GP96 C-terminal fused to Her2. Presumably, there is an optimum range and balanced level for IL-4/IFN- γ . Levels below or above that range are not efficient and fail to control tumor progression. Co-administration of GP96 with Her2 could not raise an optimum and balanced level of IL-4/IFN- γ . There was a significant difference between IL-4 level produced by GP96 C-terminal fused to Her2 compared with co-administration of GP96 with Her2 ($P<0.001$). Totally, the level of IL-4/IFN- γ produced by co-administration of GP96 with Her2 at the time of measurement was not within the optimum range required to fight tumors, and so, was not concomitant with tumor control. IL-4/IFN- γ level was measured 40 days after tumor implantation in the above-mentioned studies and the present study. On the other hand, Curcio et al. (2003) demonstrated that IFN- γ and perforin-deficient mice bearing Her2 tumors could not be cured. Our result is also consistent with them, as vaccination with GP96+Her2 could not reach the optimum level of IFN- γ and also we could not detect CTL activity by GP96+Her2 vaccine (data not mentioned).

Tregs have critical roles in tumor development and it has been reported that depletion of Tregs leads to a specific immune T cell response (Nizar et al. 2009). Such a phenomenon was observed in our experiments: a decreased percentage of Tregs was accompanied by an increased percentage of CD4+ cells at the tumor sites of pHer2+ pGP96-vaccinated animals, which could be assumed to include a specific immune T cell responder. Although, the percentage of Tregs was decreased in pGP96+pHer2-vaccinated animals and was accompanied by an increase in CD4+ effector cells, it did not coincide with control of tumor progression. This brings up the involvement and importance of other effector immune mechanisms such as antibodies. The role of antibodies on tumor growth was previously studied (Curcio et al. 2003). We did not assay antibodies raised in our setting, but we do not exclude their role in the control of tumor growth. Bi-phasic tumor growth in Fig. 3 suggests that different effector mechanisms are involved at different stage of tumor growth.

There was not a significant difference between spleen-associated Tregs in different groups. Considering the fact that 23–43% of the Tregs population are actively doubling themselves at the tumor site compared to 11–16% in spleen-associated Tregs (Lutsiak et al. 2008), partial control of tumor growth by pHer2+ pGP96 could be mediated by a decrease in the actively dividing Tregs at the tumor site, which is more than in the spleen, and therefore could be detected.

Collectively, co-administration of pGP96+pHer was effective when the tumor was small, but finally its effectiveness was inversely affected by tumor size. Such a process happened during the previous study in which HSP110/Her2 vaccine was not effective by the end of study and the tumor growth occurred later in the course of study (Manjili et al. 2003). Two reasons could be assumed: (1) failure of the immune system at the final stages or (2) because of antigen loss and tumor escape. It has been reported that IFN- γ could cause tumor escape from antitumor immune responses (He et al. 2005; Kmiecik et al. 2007). It is also presumable that there is a kinetic in regulatory burden and tumors may subvert the immune system to facilitate disease progression (Coussens and Werb 2002). Therefore, immune response should play with different cards and act dynamic at different stage of tumor. Apparently, pHer2+ pGP96 could initially overcome the regulatory burden imposed by the tumor and activate effector mechanisms. As tumor grows, different type and/or strength of effector immune responses are required to overcome the regulatory burden and eradicate tumor cells. However, effect of dose on the performance of GP96 as an adjuvant and elicitation of effective immune response was previously addressed (Liu et al. 2009). Therefore, it is notable that partial effectiveness of pHer2+ pGP96 could be because of dose effect, as we administered 100 μ g pHer2 and co-administered 50 μ g pHer2+50 μ g pGP96. Administration of 100 μ g pHer2+100 μ g pGP96 may give different result. More research needs to be performed to dissect the phenomenon occurred in our study.

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